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TITLE: The Scaffold Attachment Factor SAFB1: A New Player in G2/M Checkpoint Control

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14. ABSTRACT Loss of G2/M checkpoint plays an important role in tumorigenesis, however, few genes involved in this checkpoint control have been shown to be deregulated in human breast tumors. SAFB1 is a multifunctional protein which maps to a locus of high LOH, and mutations have been identified in both breast cancer cell lines and tumors. Our preliminary data show that inactivation of SAFB1 in MEFs result in loss of G2/M checkpoint control, and that loss of SAFB1 expression is associated with Taxotere resistance in human breast tumors. We therefore hypothesize that SAFB1 is critical for G2/M checkpoint control, and that its inactivation results in resistance to breast cancer therapies that utilize a block in G2/M and subsequent apoptosis. We will identify the mechanism by which SAFB1 controls the G2/M checkpoint, and will subsequently analyze whether Taxotere-resistant tumors show altered expression of genes involved in these pathway(s).					
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Introduction:

SAFB1 is a multifunctional protein which maps to a locus of high LOH, and mutations have been identified from both breast cancer cell lines and tumors. Our preliminary data for the grant showed that inactivation of SAFB1 in MEFs results in loss of G2/M checkpoint control, and that loss of SAFB1 expression is associated with Taxotere resistance in human breast tumors. We therefore hypothesize that SAFB1 is critical for G2/M checkpoint control, and that its inactivation results in resistance to breast cancer therapies that utilize a block in G2/M and subsequent apoptosis. We proposed to identify the mechanism(s) by which SAFB1 controls the cell cycle checkpoint(s), and to analyze whether drug-resistant tumors show altered expression of genes involved in these pathway(s).

Body:

This is the final report in which progress from Aims 1-3 is summarized according to the tasks outlined in the original statement of work.

Task 1) Is the G2/M checkpoint lost in mammary epithelial cells from the SAFB1^{-/-} knockout mice?

1.1) Grow primary mammary epithelial cells from SAFB1^{+/+} and ^{-/-} mice in culture, and expose them to γ -radiation and to agents including chemotherapeutic drugs causing mitotic defects (Taxotere, Nocodazole, Colcemid), and analyze G2/M block (FACS and apoptosis analysis). Include measurement of phospho histone H3 to more directly measure the fraction of mitotic cells (Months 1-8).

Our data showed that loss of SAFB1 was associated with a loss of G2/M checkpoint control in mouse embryonic *fibroblasts* (MEFs). We confirmed these data in MEFs, showing that these observations were made not only in the presence of Nocadazole, but also with other spindle inhibitors such as colcemid. We also exposed the cells to γ -irradiation, and, as expected, observed a decreased block in G2/M. A representative experiment is shown in Fig 1.

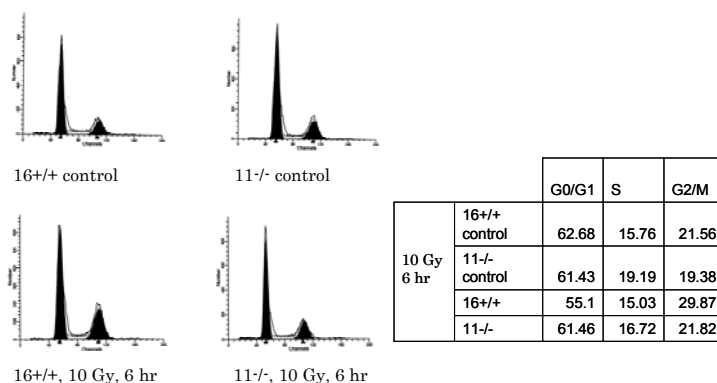


Fig 1: Decreased G2/M block in SAFB1^{-/-} MEFs. WT (16+/+ and ko (11-/-) cells were exposed to γ -irradiation (10Gy, for 6 hrs), and cell cycle distribution was determined by FACS. The data were analyzed using ModFit. Similar data were obtained with MEF pair 9^{+/+}/3^{-/-}, and irradiating for longer periods of time (up to 12 hrs). similar data obtained with another MEF pair.

As outlined in Task 1.1, we planned to extend those studies to mammary *epithelial* cells from the SAFB1^{-/-} mice. However, during year 1 of the grant we discovered that these mice were characterized by numerous pleiotropic systemic phenotypes such as low IGF1 and estrogen levels (Ivanova *et al.* 2005), which would make any interpretations of the results using the primary mammary epithelial cells very difficult. An alternative model system was human mammary epithelial cells (HMEC) in which we would decrease SAFB1 expression by shRNA technology. Unfortunately, for unknown reasons, these cells lose expression of estrogen receptor alpha (ERα) as soon as they are taken in culture, and thus do not truly represent human biology. We have spent considerable time and effort on this problem, but so far without success. We think that successful generation of such model system represents a critical problem, the solution of which will not only help us to define the role of SAFB1 in G2/M checkpoint in mammary epithelial cells, but will also be a critical tool for many other breast cancer researchers. Thus, we have decided to continue this project, and have submitted a grant proposal (“*Analysis of Corepressor Ablation in ER-Positive and ER-Negative HMECs*”) to further support these efforts to the Department of Defense Breast Cancer Research Program (BCRP).

1.2) Investigate whether SAFB1 loss leads to polyploidy and chromosome missegregation indicative of G2/M checkpoint. Therefore we will perform a number of cytogenetic studies (Months 3-8).

We tested for polyploidy by FACS analysis, and failed to detect any significant difference between wildtype and knockout MEFs. We also have complemented these studies with comparative genomic hybridization (CGH) and spectral karyotyping (SKY) studies (collaboration with Dr. Rao Pulivarthi, BCM). While our original data suggested amplification of the X-chromosome, this could not be reproduced using a number of clones. Thus, in MEFs, genetic ablation of SAFB1 did not lead to polyploidy and chromosome missegregation. This was rather unexpected and somewhat disappointing, since it indicated that SAFB1’s role in G2/M checkpoint was not as critical as we had anticipated.

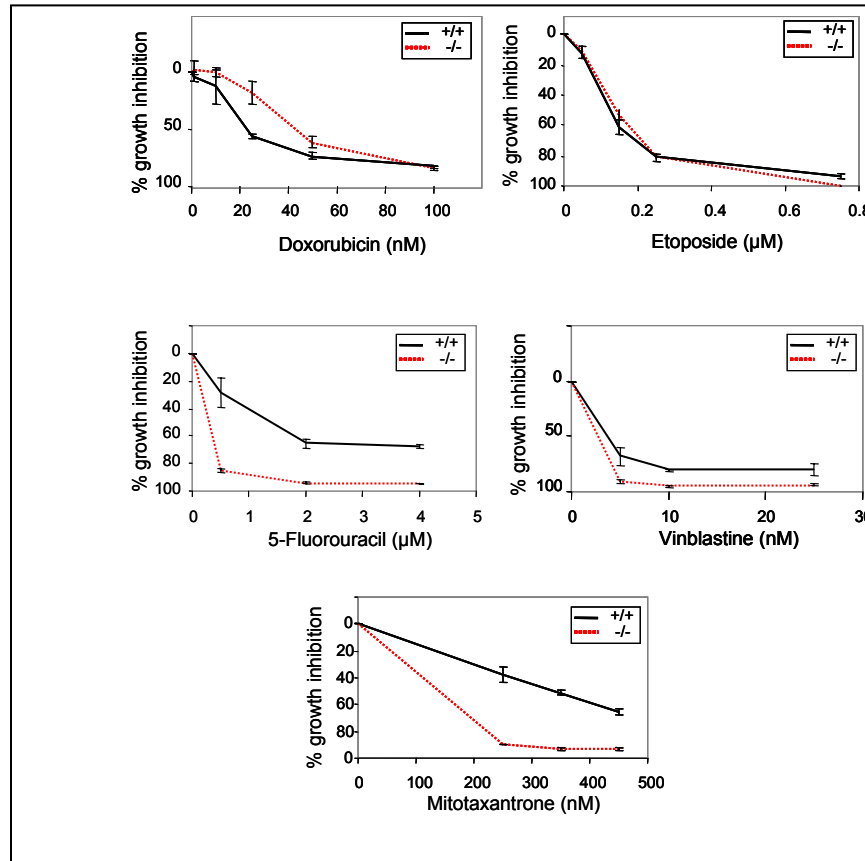
Task 2) What is the mechanism for the SAFB1-mediated G2/M checkpoint control?

2.1) Analyze the two closely linked components of the mitotic spindle, the centromere-kinetochore and the kinetochore-microtubule attachment, in SAFB1^{+/+} and SAFB1^{-/-} cells by immunofluorescence. Utilize antibodies to known centromeric and kinetochore proteins (Months 8-14).

The lack of significant effects in Task 1.2, ie no effect on polyploidy and chromosome missegregation, suggested to us that the observed association between low SAFB1 levels and increased resistance to taxotere in breast cancer patients might not be solely an effect on G2/M, but could indicate a critical role of SAFB1 in mediating apoptosis. We therefore did not immediately stain for mitotic spindle proteins, but tested whether SAFB1 loss would result in a more general loss of apoptosis. Specifically, we tested the effect of SAFB1 loss on response to various chemotherapeutic drugs (Fig 2). To our surprise, depending on drug, we observed either decreased (doxorubicin) or increased (5-FU, vinblastine, mitoxantrone) efficacy, or no effect (etoposide). We conclude that loss of SAFB1 is not associated with a general drug resistance, but rather is a differential modulator of chemotherapy-induced apoptosis. Interestingly, very similar findings have been described for BRCA1, where the response also depends on the nature of the cellular insult (Quinn *et al.*, 2003). Thus, the SAFB1 loss-associated resistance to

docetaxel does not seem to be the result of a general loss of G2/M checkpoint control since we did not observe resistance to other drugs effecting the mitotic spindle such as vinblastine.

Figure 2. Analysis of SAFB1^{-/-} MEFs' sensitivity to various chemotherapeutic drugs. MEFs stably transformed with Ras/Myc were incubated in the presence of various drugs at indicated concentrations, and growth was measured on day 3. A representative clone is shown. Each data point represents average of a triplicate well.



Interestingly, the resistance to the G2/M drug docetaxel in SAFB1^{-/-} MEFs was dependent upon c-myc overexpression — SAFB1 ko MEFs transformed with ras/myc but not with ras/SV40T show increased resistance to the effects of docetaxel. This finding was especially interesting in light of our critical observations that SAFB1 ko MEFs spontaneously immortalize (Fig 3) which was associated with a loss of the tumor suppressor and cell cycle inhibitor p19ARF (Wadhwa *et al.* 2004) (Fig 3). We published these findings in *Cancer Research* last year (Dobrzycka *et al.*, 2006). Since p19ARF has been

shown to be critical for myc-induced apoptosis (Cleveland *et al.* 2004, Qi *et al.* 2004, Zindy *et al.* 1998), these data further support the idea that loss of SAFB1 results in docetaxel resistance due to loss of myc-induced apoptosis.

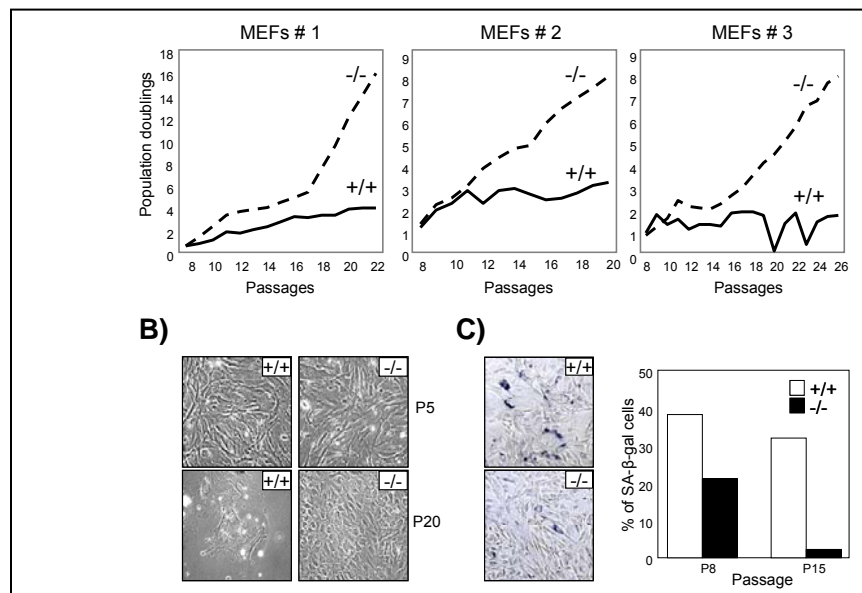


Figure 3. SAFB1^{-/-} MEFs fail to undergo senescence and spontaneously immortalize. A) Growth of MEFs passaged according to the 3T3 protocol. The graphs represent the accumulated number of population doublings of MEF pairs from three different mothers. B) Representative phase contrast images of MEFs at passages 5 and 20 (left panel). C) Expression of senescence-associated β-galactosidase (SA-β-gal) in MEFs at passage 8 (left

panel). The percentage of β -gal positive MEFs was calculated and graphed for each genotype for passage 8 and 15 (right panel).

2.2) *Determine which SAFB1 domains are necessary and sufficient for G2/M arrest. Compare how re-introduction of wildtype SAFB1 and SAFB1 mutants lacking DNA-binding and protein-protein interaction domains restores the G2/M checkpoint in SAFB1^{-/-} cells (Months 13-18).*

Due to some technical reasons, this task took longer than originally anticipated. However, we have now finished generating clones that stably overexpress various SAFB1-deletion constructs (Fig 4). Although the grant has finished, we expect to be able to screen these clones shortly for altered response to chemotherapeutic drugs. This is critical for our understanding of SAFB1's role in drug resistance.

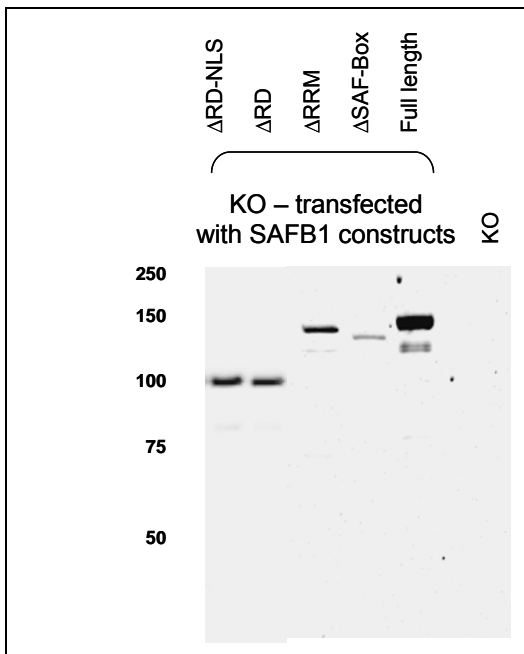


Fig 4: Generation of KO MEFs over-expressing full-length or various SAFB1 deletion mutants. Δ RD-NLS – deletion of repression domain but including nuclear localization signal; Δ RD-NLS – deletion of repression domain; Δ RRM – deletion of RNA recognition domain; Δ SAF-Box – deletion of scaffold attachment factor box, the DNA binding domain. The picture represents immunoblot using anti-HA antibodies since all constructs were HA-tagged.

2.3) *Identify SAFB1-interacting proteins which participate in the same spindle checkpoint pathways through immunoprecipitation. Concentrate on proteins which specifically interact with SAFB1 in mitosis. Use antibodies to known proteins to identify potential candidate proteins (Months 13-20).*

AND

2.4) *Perform protein sequencing of unknown proteins from Task 2.3 (Months 18-24).*

During the time of the grant proposal, another group identified SAFB1-interacting proteins in cancer cells through co-immunoprecipitation studies, followed by mass spectrometry (the complete data are available on the webpage <http://www.nursa.org/>). This analysis did not identify any members of the spindle checkpoint. However, coincidentally we were contacted by Dr Thomas Giannakouris (Department of Chemistry, Aristotle University of Thessaloniki, Greece). Drs Giannakouris and Scott have discovered that SAFB1 interacts with the proliferation potential protein-related P2P-R gene product. Interestingly, P2P-R is highly expressed in mitotic cells, and localizes to the periphery of chromosomes in mitotic cells (Gao *S et al.*, 2002). P2P-R overexpression inhibits mitosis, and promotes mitotic apoptosis. Interestingly, P2P-R has been shown to be associated with response to various drugs including

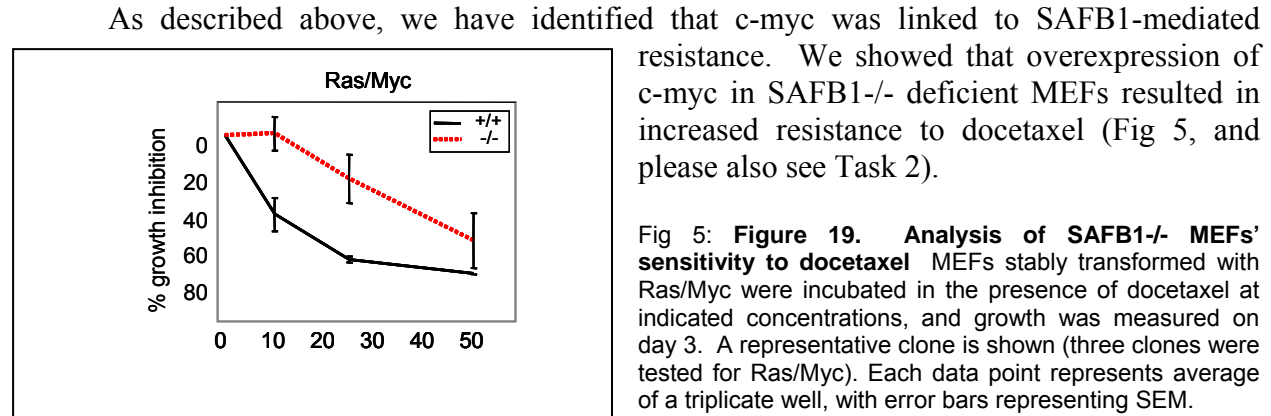
camptothecin and nocadazole (Gao and Scott, 2003). We have therefore started a collaboration with Dr Giannakourus laboratory, have received expression constructs, and have begun to study the interactions between those two proteins, and its functional relevance. Since this collaboration has only started a few months ago, we have not yet received any conclusive results.

3) Are genes which are mechanistically linked to SAFB1-mediated checkpoint control also deregulated in Taxotere-resistant breast cancer?

3.1) Analyze whether inactivation of candidate genes (identified in Aim 2.3) also leads to Taxotere resistance in cell line models. Depending on availability either enter collaborations with other investigators to study the genes in preexisting models, or use RNAi technology to inactivate them. Analyze G2/M checkpoint defects and Taxol resistance (as in Task 1) (Months 22-28).

AND

3.2) Analyze expression of these genes in Taxotere resistant breast tumors. If the genes are present on the Affymetrix Chip used for the original neoadjuvant study, reanalyze the data with respect to changes of the expression of these genes. Otherwise, stain fixed tissues if antibodies are available, or do quantitative RT-PCR studies using RNA from breast tissues. (Months 25-36).



We therefore decided to re-analyze the original data from the gene expression analysis using biopsies from breast cancer patients treated with docetaxel (Chang *et al.* 2003). Briefly, for this study, RNA was obtained from locally advanced breast tumors in 24 patients before they underwent four cycles of neoadjuvant docetaxel treatment. Gene expression analysis was performed and correlated to the treatment response to determine genes that are differentially expressed in docetaxel-sensitive and -resistant tumors. Using this microarray database, supervised analysis, performed by Dr. Anna Tsimelzon (Breast Center Biostatistics Core, Baylor College of Medicine), had previously shown that SAFB1 levels were significantly lower in docetaxel-resistant tumors as compared to the sensitive tumors ($p = 0.0001$).

We were now interested in looking at P2P-R and c-myc on this data set. Unfortunately, P2P-R was not on the arrays used for the study (U95Av2) and could therefore not be evaluated.

We repeated the same study with c-myc, and found that its levels were significantly higher in the docetaxel-resistant, as compared to the sensitive tumors ($p = 0.0055$) (Fig 6). Notably, SAFB1 and Myc expression levels were strongly and significantly inversely correlated (Pearson correlation coefficient $r = -0.74$) in these tumors from patients. Thus, these clinical data are consistent with our findings from the transformed MEFs. Future studies will be aimed at understanding the nature of the inverse correlation between SAFB1 and c-myc expression.

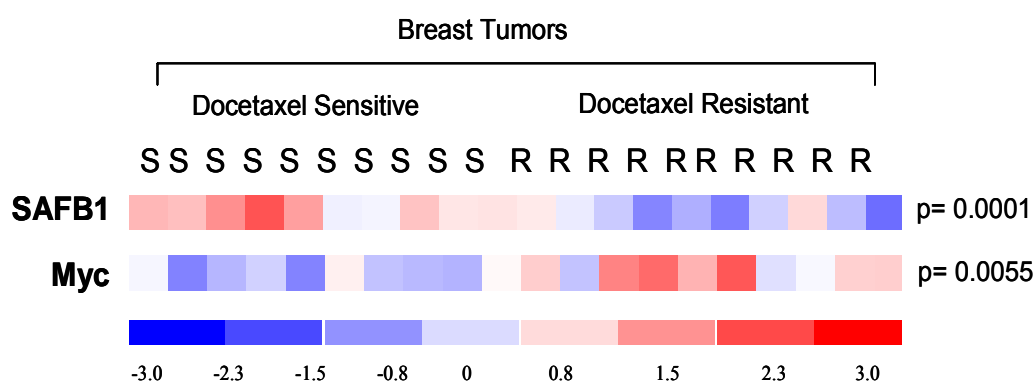


Figure 6. Analysis of SAFB1 levels in docetaxel resistant breast tumors. Analysis of SAFB1 and Myc levels in docetaxel-sensitive (S) and -resistant (R) breast cancer samples. The red color represents expressions level above mean expression of a gene, and the blue color represents expression lower than the mean. The color scale on the bottom represents standardized expressions. Each gene is standardized to have mean 0 and standard deviation 1.

Key Research Accomplishments:

- 1) Association of SAFB1 levels with increased or decreased sensitivity to drugs, depending on the chemotherapeutic agent used
- 2) Association of SAFB1 loss with resistance to docetaxel treatment, both in cell line models and in patient material
- 3) SAFB1 loss was not associated with polyploidy and chromosome missegregation, suggesting a moderate effect of SAFB1 on G2/M checkpoints
- 4) Spontaneous immortalization of SAFB1 KO MEFs, and loss of senescence, which was associated with loss of induction of cell cycle inhibitor p19^{ARF}
- 5) Inverse correlation of SAFB1 and myc in breast cancer samples

Reportable Outcomes

Poster Presentation:

Dobrzycka KM, Kang K, Jiang S, Meyer R, Chang J, Tsimelzon A and Oesterreich S. Disruption of scaffold attachment factor B1 leads to cell immortalization and docetaxel resistance. 3rd Annual Cancer Center Symposium. Houston, TX. October 28, 2005.

Dobrzycka KM, Kang K, Ivanova M, Jiang S, and Oesterreich S. Loss of SAFB1 leads to a tumorigenic phenotype of Mouse Embryo Fibroblasts (MEFs). Era of Hope 2005 Department of Defense Breast Cancer Research Program Meeting. Philadelphia, PA. June 8-11, 2005.

Dobrzycka KM, Kang K, Ivanova M, Jiang S, Rao P, Lee AV, Oesterreich S. SAFB1 as a tumor suppressor: its role in immortalization, transformation, and genomic instability. 27th Annual San Antonio Breast Cancer Symposium. San Antonio, TX. December, 2004.

Oral Presentation:

Dobrzycka KM, Oesterreich S. SAFB1 As a Novel Tumor Suppressor Gene in Breast Cancer. Gordon Research Conference: Cancers Models and Mechanisms. Newtown, RI. August 2004. (Oral presentation)

Manuscripts:

Klaudia M. Dobrzycka, Kaiyan Kang, Shiming Jiang, Rene Meyer, Rao Pulivarthi, Adrian V. Lee, and Steffi Oesterreich. Disruption of Scaffold Attachment Factor SAFB1 Leads to TBX2 up-regulation, Lack of p19^{ARF} Induction, Lack of Senescence, and Cell Immortalization. *Cancer Research* 66: 7859-7863, 2006.

A second manuscript which will focus on effect of SAFB1 loss on altered sensitivity to various chemotherapeutic drugs is currently in preparation. We expect to submit this paper within the next 2-4 months.

Pending Grant:

“Analysis of Corepressor Ablation in ER-Positive and ER-Negative HMECs”. Submitted to Department of Defense Breast Cancer Research Program

Collaborations

We have begun a collaboration with Dr Thomas Giannakouras (Department of Chemistry, Aristotle University of Thessaloniki, Greece), who is studying the P2P-R gene product. Published data and recent unpublished observation suggest that SAFB1 and P2P-R functionally interact.

Conclusions:

We have confirmed that loss of SAFB1 results in resistance to Taxotere (docetaxel), and have determined that this resistance can especially be observed in cells which also overexpress myc. We have found that there is a strong inverse relationship between SAFB1 and c-myc in breast tumors, the reason for which is currently unknown. During these studies, we have also made the exciting observation that SAFB1 null cells spontaneously immortalize which was associated with loss of p19ARF. Together with our previous results, these data suggest that loss of SAFB1 might result in loss of apoptosis, since p19ARF is a critical mediator of myc-induced apoptosis. This hypothesis is further supported by the observations that a) loss of SAFB1 was not associated with polyploidy or major defects in chromosome segregation, and b) that loss of

SAFB1 with increased or decreased sensitivity to drugs, depending on the chemotherapeutic agent used.

Future studies are aimed at understanding the functional relevance of P2P-R/SAFB1 interactions, and whether loss of SAFB1 in HMECs will also result in loss of senescence and altered drug sensitivity. We have already submitted one grant to further support these studies, and expect to submit at least one additional within the next year.

References:

- 1) Chang JC, Wooten EC, Tsimelzon A, Hilsenbeck SG, Gutierrez MC, Elledge R, Mohsin S, Osborne CK, Chamness GC, Allred DC, *et al.* 2003 Gene expression profiling for the prediction of therapeutic response to docetaxel in patients with breast cancer. *Lancet* **362** 362-9
- 2) Cleveland JL & Sherr CJ 2004 Antagonism of Myc functions by Arf. *Cancer Cell* **6** 309-11.
- 3) Gao S, Scott RE. 2003 Stable overexpression of specific segments of P2P-R protein in human MCF-7 cells promotes camptothecin-induced apoptosis. *J Cell Physiol* **197** 445-52
- 4) Gao S, Scott RE. 2002. P2P-R overexpression restricts mitotic progression at prometaphase and promotes mitotic apoptosis. *J Cell Physiol* **193** 19-207
- 5) Gao S, Witte MM, Scott RE 2002 P2P-R protein localizes to the nucleolus of interphase cells and the periphery of chromosomes in mitotic cells which show maximum P2P-R immunoreactivity. *J Cell Physiol* **191** 145-154
- 6) Ivanova M, Dobrzycka KM, Jiang S, Michaelis K, Meyer R, Kang K, Adkins B, Barski OA, Divisova J, Lee AV, Oesterreich S. Scaffold Attachment Factor B1 functions in development, growth, and reproduction. *Molecular and Cellular Biology*, **25**:2995-3006, 2005.
- 7) Qi Y, Gregory MA, Li Z, Brousal JP, West K & Hann SR 2004 p19ARF directly and differentially controls the functions of c-Myc independently of p53. *Nature* **431** 712-7. Epub 2004 Sep 8.
- 8) Quinn JE, Kennedy RD, Mullan PB, Gilmore PM, Carty M, Johnston PG, Harkin DP. 2003 BRCA1 functions as differential modulator of chemotherapy-induced apoptosis. *Cancer Research* **63** 6221-8
- 9) Schmeichel KL & Bissell MJ 2003 Modeling tissue-specific signaling and organ function in three dimensions. *Journal of Cell Science* **116** 2377-88
- 10) Wadhwa R, Sugihara T, Taira K & Kaul SC 2004 The ARF-p53 senescence pathway in mouse and human cells. *Histology and Histopathology* **19** 311-6.
- 11) Zindy F, Eischen CM, Randle DH, Kamijo T, Cleveland JL, Sherr CJ & Roussel MF 1998 Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes and Development* **12** 2424-33.

Appendix

1) List of Personnel receiving pay from the research effort

Steffi Oesterreich, PhD
Kaiyan Kang
William Mansfield
Marijke Schrock

2) Published manuscript

Klaudia M. Dobrzycka, Kaiyan Kang, Shiming Jiang, Rene Meyer, Rao Pulivarthi, Adrian V. Lee, and Steffi Oesterreich. Disruption of Scaffold Attachment Factor SAFB1 Leads to TBX2 up-regulation, Lack of p19^{ARF} Induction, Lack of Senescence, and Cell Immortalization. *Cancer Research* 66: 7859-7863, 2006.

Disruption of Scaffold Attachment Factor B1 Leads to TBX2 Up-regulation, Lack of p19^{ARF} Induction, Lack of Senescence, and Cell Immortalization

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Abstract

Scaffold attachment factor B1 (SAFB1) is a multifunctional protein, which has previously been implicated in breast cancer. Here, we show that genetic deletion of SAFB1 in mouse embryonic fibroblasts (MEF) leads to spontaneous immortalization and altered expression of two proteins involved in immortalization and escape from senescence: low levels of p19^{ARF} and high levels of TBX2. Inactivation of TBX2 using a dominant-negative TBX2 resulted in up-regulation of p19^{ARF} in SAFB1 knockout MEFs. SAFB1 loss also caused lack of contact inhibition, increased foci formation, and increased oncogene-induced anchorage-independent growth. These findings suggest that SAFB1 is a novel player in cellular immortalization and transformation. (Cancer Res 2006; 66(16): 7859-63)

Introduction

Scaffold attachment factor B1 (SAFB1) is a nuclear protein involved in RNA processing, transcriptional regulation, chromatin organization, and stress response (1). The protein contains numerous highly conserved functional domains. SAFB1 can bind RNA via a RNA recognition motif (2) and is found in complexes with RNA processing proteins (3, 4). Because SAFB1 can also interact with RNA polymerase II, it has been suggested to be part of a "transcriptosome" complex (3). The NH₂ terminus harbors a SAF-Box, a homeodomain-like DNA-binding motif that interacts with scaffold/matrix attachment regions (S/MAR; ref. 5). In addition, an independent repression domain is located at the COOH terminus (6).

Previous studies suggested that SAFB1 plays an important role in human breast cancer. It functions as an estrogen receptor α (ER α) corepressor by directly binding to ER α and inhibiting its transcriptional activity (7). SAFB1 maps to a chromosomal locus that displays unusually high rates of loss of heterozygosity in invasive breast cancers, and SAFB1 mutations have been identified in microdissected breast tumor tissue but not in the normal adjacent tissue (8).

We have recently generated SAFB1 knockout mice, which show high preneonatal and neonatal lethality, severe dwarfism associated with low insulin-like growth factor-I levels, and female subfertility and male infertility (9). Here, we show that mouse embryonic

fibroblasts (MEF) from SAFB1^{-/-} mice fail to undergo senescence and exhibit spontaneous immortalization, which was associated with a lack of p19^{ARF} induction and high levels of TBX2, a known p19^{ARF} repressor. SAFB1^{-/-} cells proliferate in growth-restricting conditions and show increased anchorage-independent growth in the presence of cooperating oncogenes. These findings place SAFB1 in a unique group of genes that are critical in cellular immortalization and transformation.

Materials and Methods

Cells and tissue culture. MEF pairs were obtained from sibling SAFB1^{+/+} and SAFB1^{-/-} embryos from the same mother as described previously (9), and experiments were repeated using other MEF pairs from different mothers. Short-term proliferation assays were done by plating 1×10^4 cells per well in 24-well plates in triplicates, and cells were counted daily. For foci formation assays, MEFs were plated at 1×10^6 in a 10-cm plate and cells were transiently transfected using Lipofect-AMINE (Invitrogen, Carlsbad, CA) with oncogenes. Medium was changed twice weekly, and foci were allowed to form for 2 to 3 weeks. To obtain stably transformed MEFs, cells were transfected and single colonies were expanded. To test anchorage-independent growth, MEFs were suspended in 0.4% SeaPlaque agar (Cambrex, East Rutherford, NJ) in the growth medium and overlaid on 0.8% agar in the same medium. Colonies were stained with 1 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (ICN, Irvine, CA) 2 weeks later and counted. Population doublings were calculated according to the formula $\log(\text{final cell number}/\text{plated cell number}) / \log_2$. Senescence-associated β -galactosidase (SA- β -gal) was measured by using a Senescence β -galactosidase kit (Cell Signaling, Beverly, MA).

Protein and RNA analysis. Immunoblotting was done as described previously (2). The p19^{ARF} antibody was purchased from Abcam (Cambridge, United Kingdom), and the antibody recognizing mouse SAFB1 has been described previously (9).

For the reverse transcription-PCR (RT-PCR) analysis, total cellular RNA was isolated with RNeasy kit (Qiagen, Valencia, CA), RNA was reverse transcribed with SuperScript II RNase H Reverse Transcriptase (Invitrogen), and PCR was done using primers used for SAFB1 (9), p19^{ARF} (10), TBX2 (11), Bmi-1 (5'-TGTGTCCTGTGTGGAGGTA-3' and 5'-TGGTTTGTGAACCTGGACA-3'), CBX7 (5'-TGTCAGCCATAGGCGAGCA-3' and 5'-AACTTTGCCCTTCCGACAG-3'), and DMP1 (5'-CTGTAGCTGAAAGAGTGGGTA-3' and 5'-TGTATTATCTTCCAAGCGGGC-3'). To measure TBX2 and TBX3 mRNA by quantitative PCR using an ABI PRISM 7700 (PE Applied Biosystems, Foster City, CA), RNA was DNase treated and reverse transcribed and cDNA was amplified for 40 cycles of 95°C for 12 seconds and 60°C for 1 minute. The change in expression was calculated by the $\Delta\Delta C_t$ method. The primers (mouse TBX2, 5'-TGAAGCTCCATACAGCACCTT-3' and 5'-TTGTGCGATCTTCAGCTGTGTAATCT-3'; mouse TBX3, 5'-CCACCTCCAACAACACGTTCT-3' and 5'-TAAGGAAACAGGCTCCCGAA-3'; and mouse β -actin, 5'-GCTCTGGCTCCTAGCACCAT-3' and 5'-CCACCGATCCACACAGAGTAC-3') and Taqman probes (TBX2, TCTTCCAGAGACCGACTTCATCGCTG; TBX3, CCTCCAGGGCTTGGCTATGTCGC; and β -actin, ATCAAGATCATGTCTCTCTGAGCGC) were purchased from

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Eurogentec (Philadelphia, PA) or Integrated DNA Technologies, Inc. (Coralville, IA).

Virus production and MEFs infection. 293T cells were transfected with ecotropic helper virus DNA (pCL-Eco) and either pBabe-p19^{ARF}, pBabe-rasV12, pBabe-control, or pBabe-dnTBX2 (12). Supernatants containing infectious retrovirus were harvested 24 to 72 hours after transfection, filtered, and stored on ice. MEFs were infected thrice for 3 to 4 hours each with virus-containing supernatant containing 10 µg/mL polybrene (Sigma, St. Louis, MO).

Results

SAFB1 deficiency leads to lack of senescence and immortalization. To analyze the effect of SAFB1 loss on tumorigenesis *in vitro*, we generated pairs of SAFB1^{+/+} and SAFB1^{-/-} MEFs and confirmed SAFB1 status by RT-PCR and Western blot (Fig. 1A). Growth curves using early passage MEFs revealed slightly slower growth of SAFB1^{-/-} MEFs compared with SAFB1^{+/+} MEFs (Fig. 1B).

Next, we determined the effect of SAFB1 loss on cellular senescence and immortalization by long-term passaging of MEFs obtained from three independent SAFB1^{+/+} and SAFB1^{-/-} embryos following the 3T3 protocol. As expected, SAFB1^{+/+} MEFs showed a decline in their proliferative rate at about passage 12 and ultimately underwent senescence (Fig. 1C). Of three SAFB1^{+/+}

MEFs analyzed, only one immortal population emerged after passage 28. After several attempts, we were also able to generate immortalized populations from the other two SAFB1^{+/+} MEFs. In contrast, all the SAFB1^{-/-}-independent MEFs did not lose proliferative capacity following serial passaging with increased proliferative potential that emerged at passages 12 to 16. This bypass of cellular senescence was easily observed microscopically. Early passage, exponentially growing SAFB1^{+/+} and SAFB1^{-/-} MEFs did not differ in cell morphology (Fig. 1D). In contrast, late passage SAFB1^{+/+} acquired a large, flattened cell morphology, characteristic of senescent cells, whereas SAFB1^{-/-} MEFs remained small and more refractile. This morphology change, together with the biphasic curves for the population doublings (MEF pairs 1 and 3), suggests that there is an outgrowth of a specific subgroup of MEFs. We further confirmed the lack of senescence by staining for SA-β-gal. Whereas SAFB1^{+/+} MEFs showed high levels of SA-β-gal activity, SAFB1^{-/-} MEFs showed decreased SA-β-gal activity at passages 8 and 15 (Fig. 1E). These data indicate that loss of SAFB1 results in immortalization by allowing MEFs to bypass cellular senescence.

Immortalization of SAFB1^{-/-} MEFs is associated with lack of p19^{ARF} induction and increased TBX2 levels. Next, we measured levels of p19^{ARF}, a known inducer of senescence in MEFs

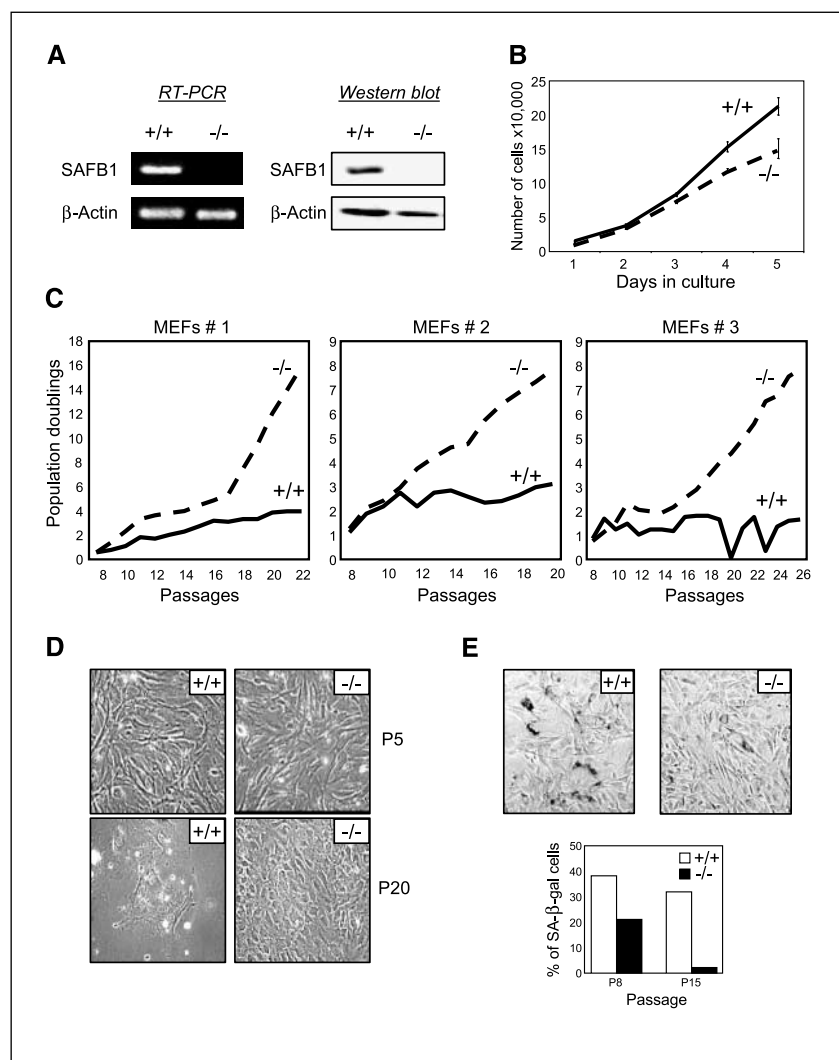


Figure 1. SAFB1^{-/-} MEFs fail to undergo senescence and spontaneously immortalize. **A**, RT-PCR and immunoblot analysis of SAFB1^{+/+} and SAFB1^{-/-} MEFs. β-Actin was used as a loading control. **B**, growth curve of primary MEFs (passage 5). Points, cell count from triplicate wells; bars, SE. Similar results were obtained with at least three independent MEF pairs. **C**, growth of MEFs passaged according to the 3T3 protocol. Accumulated number of population doublings of MEF pairs from three different mothers. **D**, left, representative phase-contrast images of MEFs at passages 5 and 20. **E**, top, expression of SA-β-gal in MEFs at passage 8; bottom, percentage of β-gal-positive MEFs was calculated and graphed for each genotype for passages 8 and 15.

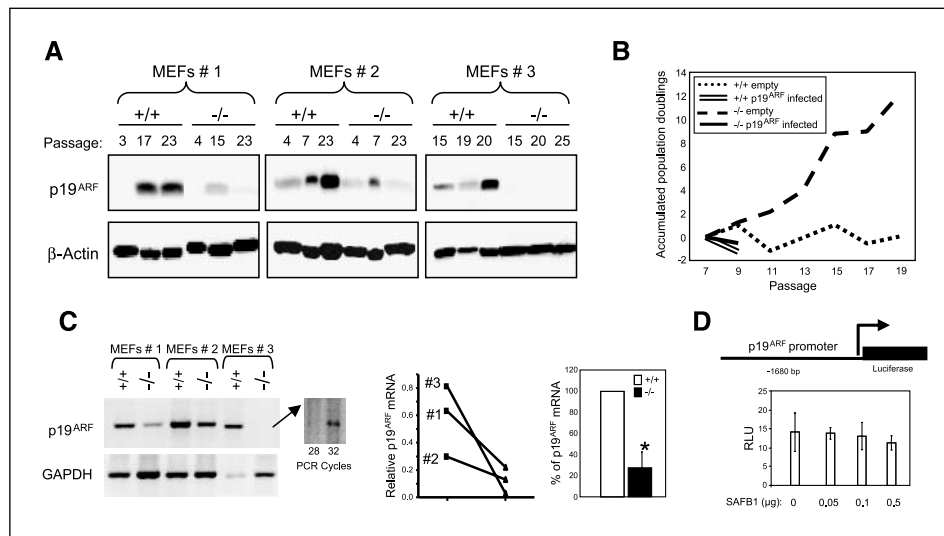
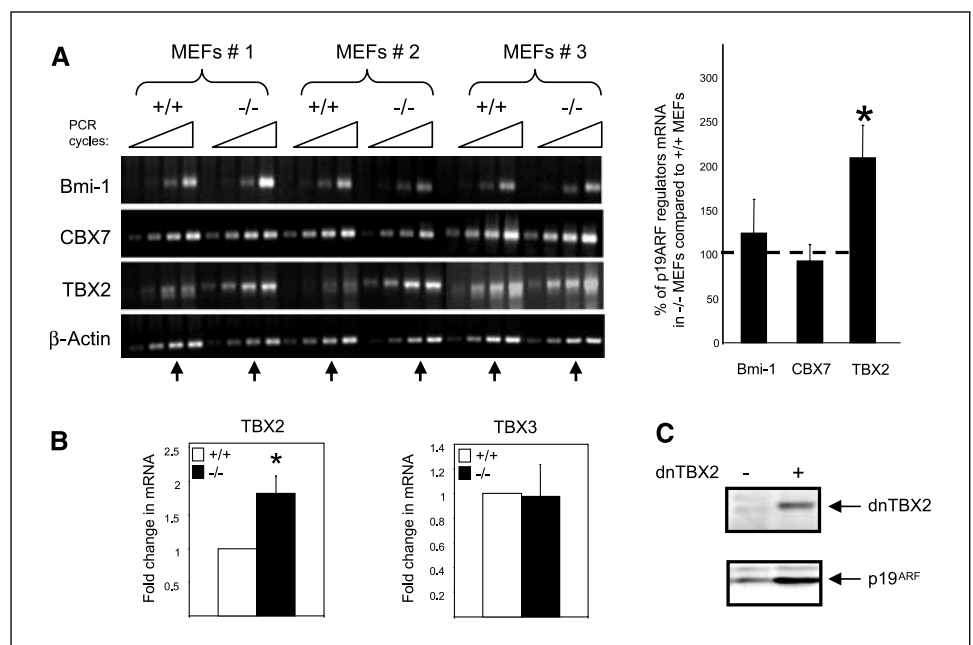


Figure 2. Lack of p19^{ARF} induction in SAFB1^{-/-} MEFs. **A**, immunoblot analysis of p19^{ARF} using protein lysates from three individual MEF pairs at indicated passages. β -Actin was used as loading control. **B**, reintroduction of p19^{ARF} into SAFB1^{+/+} and SAFB1^{-/-} MEFs. Cells at passage 7 were infected with virus delivering empty vector or p19^{ARF} and serially passaged following the 3T3 protocol. **C**, p19^{ARF} mRNA expression in SAFB1^{+/+} and SAFB1^{-/-} MEFs. p19^{ARF} was amplified from three MEF pairs [corresponding to (A)] by RT-PCR (26 cycles). For SAFB1^{-/-} MEF pair 3, the PCR cycle number needed to be increased to 32 to detect the PCR product. Graphical representation of densitometry from the RT-PCR shows relative p19^{ARF} mRNA [corrected for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)] in a paired and nonpaired *t* test. *, *P* = 0.047. **D**, MEFs were transfected with a p19^{ARF}-Luc (-1,680 bp) reporter construct and SAFB1pcDNA1 as indicated, and relative luciferase unit (*RLU*) was determined.

(Fig. 2A; ref. 13). As expected, p19^{ARF} levels were induced with increased passaging of SAFB1^{+/+} MEFs. In contrast, we found unusually low p19^{ARF} levels in SAFB1^{-/-} MEFs; pairs 1 and 2 showed very weak p19^{ARF} induction and no p19^{ARF} protein was detected in pair 3 even after prolonged exposure. Reintroduction of p19^{ARF} into SAFB1^{-/-} cells by retroviral infection resulted in growth arrest, whereas cells infected with empty virus maintained constant growth and did not undergo senescence (Fig. 2B). These findings strongly suggest that the lack of senescence in SAFB1^{-/-} cells was due to lack of p19^{ARF} induction and that other downstream effectors of p19^{ARF} were not inactivated.

To determine whether p19^{ARF} protein changes were also reflected by changes at the mRNA level, we measured p19^{ARF} mRNA and detected significant decreases in all three SAFB1^{-/-} MEFs compared with SAFB1^{+/+} (Fig. 2C). In SAFB1^{-/-} MEF pair 3, which did not express p19^{ARF} protein, we detected very low mRNA, suggesting that the genomic locus was intact in all SAFB1^{-/-} MEFs. We did not detect any effect of SAFB1 on a p19^{ARF} promoter (-1,680 bp) activity using transient reporter assays in MEFs (Fig. 2D) and in MCF-7 cells (data not shown), suggesting that it was unlikely that SAFB1 directly regulates expression of p19^{ARF} transcription. We therefore hypothesized that SAFB1 may indirectly

Figure 3. Increased TBX2 levels in SAFB1^{-/-} MEFs. **A**, expression levels of repressors of p19^{ARF} levels in three independent MEF pairs. Semiquantitative RT-PCR was done for Bmi-1, CBX7, and TBX2 using increasing number of PCR cycles (Bmi-1, 15, 18, 21, and 24; CBX7, 30, 33, 36, and 39; and TBX2, 29, 32, 35, and 38) with β -actin (cycle numbers 15, 18, 21, and 24) as a control. Arrow, band intensities were quantified at one cycle. Ratio of SAFB1^{-/-} to SAFB1^{+/+}. Columns, mean of three independent MEF pairs; bars, SD. *, *P* = 0.0078, two-tailed *t* test. **B**, analysis of TBX2 and TBX3 mRNA in primary SAFB1^{-/-} MEFs. TBX2 and TBX3 mRNA levels in MEFs (*n* = 6) were measured by quantitative PCR. Relative mRNA levels corrected for β -actin. *, *P* = 0.0281, two-tailed paired *t* test. Columns, mean (*n* = 6); bars, SE. **C**, dominant-negative TBX2 (*dnTBX2*) up-regulates p19^{ARF} expression. SAFB1^{-/-} MEFs were infected with pBabe-dnTBX2, and protein expression of dominant-negative TBX2 and p19^{ARF} was analyzed using hemagglutinin and p19^{ARF} antibodies, respectively.



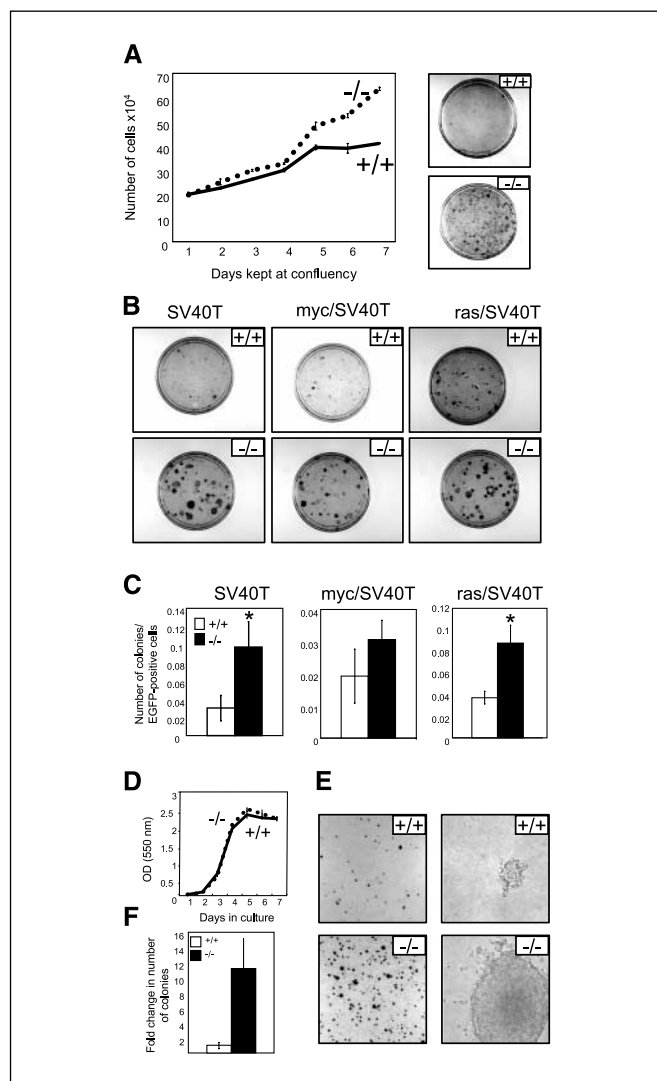


Figure 4. Lack of SAFB1 leads to a partially transformed phenotype. **A**, postconfluent growth of MEFs. *Left*, SAFB1^{+/+} and SAFB1^{-/-} MEFs (passage 7) were plated at confluency, and growth was measured. *Points*, mean of experiments done in triplicate; *bars*, SE. Similar results were obtained in two independent experiments. *Right*, SAFB1^{+/+} and SAFB1^{-/-} MEFs (passage 10) were kept at confluency for 3 to 4 weeks. Pictures are representative of three independent experiments. **B**, anchorage-dependent, oncogene-induced (SV40T, myc/SV40T, and ras/SV40T) foci formation assay in primary MEFs. Similar results were obtained in at least three independent experiments. **C**, quantitative analysis of anchorage-dependent, oncogene-induced (SV40T, myc/SV40T, and ras/SV40T) foci formation assay in primary MEFs ($n = 3$ per genotype). *Columns*, mean foci number of three clones corrected for number of fluorescent cells (to correct for transfection efficiency); *bars*, SE. *, $P < 0.05$. **D**, growth properties of MEFs stably transformed with ras/SV40T. Representative growth curves for three independent transformed MEF clones for each genotype. *Points*, mean of experiments done in triplicate; *bars*, SE. **E**, anchorage-independent growth of SAFB1^{+/+} and SAFB1^{-/-} MEFs stably transformed with ras/SV40T. Pictures at low (*left*) and high (*right*) magnifications. **F**, *columns*, mean of three independent clones shown as fold change compared with SAFB1^{+/+}; *bars*, SE.

affect p19^{ARF} expression by inhibiting a p19^{ARF} repressor, such as Bmi-1 (14), CBX7 (15), or TBX2 (16). RT-PCR analysis revealed that there was no difference in expression of Bmi-1 and CBX7; however, TBX2 was significantly increased in SAFB1^{-/-} MEFs (Fig. 3A). TBX2 overexpression was confirmed by quantitative PCR using additional MEF pairs (Fig. 3B). Expression of TBX3, a gene that has also been shown to regulate p19^{ARF} and senescence (17),

was not altered (Fig. 3B), suggesting that SAFB1 specifically regulates expression of TBX2. This regulation was not at the level of RNA stability because we did not detect a difference in TBX2 RNA half-life between SAFB1^{+/+} and SAFB1^{-/-} MEFs (data not shown).

Overexpression of a dominant-negative TBX2, previously described by Vance et al. (12), into SAFB1^{-/-} MEFs resulted in up-regulation of p19^{ARF} (Fig. 3C). Together, these findings provide support for a model, in which SAFB1 loss results in increased TBX2 expression associated with lack of induction of p19^{ARF} and thus lack of senescence and spontaneous immortalization.

Loss of SAFB1 leads to features of transformed phenotype.

Attainment of cell immortality is a prerequisite for transformation (18), and several MEF models, which undergo spontaneous immortalization, also display increased transformation capabilities. To determine whether SAFB1^{-/-} MEFs showed increased transformation characteristics, we tested their ability to proliferate in growth-restricting conditions. When cells were grown at confluency, SAFB1^{+/+} MEFs showed contact inhibition and were growth arrested (days 5–7), whereas SAFB1^{-/-} MEFs continued to proliferate and achieved a higher density cellular monolayer (Fig. 4A, *left*). This loss of contact inhibition was even more pronounced when confluent cells were cultured long term (4 weeks), resulting in formation of multiple foci in SAFB1^{-/-} MEFs (Fig. 4A, *right*).

MEFs lacking p19^{ARF} fail to undergo senescence provoked by constitutive signaling of oncogenic ras (19) but are instead transformed. Infection of SAFB1^{-/-} MEFs with rasV12 did not result in full transformation (data not shown); thus, loss of SAFB1 alone does not cooperate with ras in cellular transformation. In contrast, overexpression of SV40T, myc/SV40T, or ras/SV40T resulted in increased number and size of foci (Fig. 4B), suggesting that SAFB1 loss results in increased efficiency of transformation by cooperating oncogenes. To determine whether number of foci was also increased in SAFB1^{-/-} MEFs, we cotransfected the oncogenes with enhanced green fluorescent protein (EGFP) and corrected the numbers of foci for the number of EGFP-positive cells. Overexpression of SV40T and ras/SV40T resulted in increased number of foci as shown in Fig. 4C.

We also generated stable clones of ras/SV40-transformed SAFB1^{-/-} and SAFB1^{+/+} cells. There was no difference in anchorage-dependent growth between ras/SV40T-transformed SAFB1^{-/-} and SAFB1^{+/+} cells (Fig. 4D; $n = 3$ per genotype). However, anchorage-independent growth in soft agar was dramatically increased as a consequence of SAFB1 loss in the stable clones (Fig. 4E and F). These data provide further evidence that loss of SAFB1 results in increased transformed phenotypes.

Discussion

Here, we provide critical data about the role of SAFB1 in cellular immortalization and transformation. MEFs lacking SAFB1 show altered expression of two proteins involved in the senescence and immortalization processes: low levels of p19^{ARF} and high levels of TBX2, a known repressor of p19^{ARF}. Although SAFB1^{-/-} MEFs are not fully transformed, they are able to proliferate in growth-restricting conditions and show increased anchorage-independent growth in the presence of oncogenes.

The failure of SAFB1^{-/-} MEFs to senesce and, as a consequence, to undergo spontaneous immortalization places SAFB1 into a unique set of genes regulating this process (20). We show that loss of SAFB1 leads to a reduction in p19^{ARF} levels but not to a

complete abrogation of expression. This effect was not direct, but indirect, possibly caused by increased expression of TBX2. TBX2 is a T-box transcription factor, which has been shown to repress p19^{ARF} promoter activity and whose overexpression leads to cell immortalization (16, 17). Indeed, inactivation of TBX2 using a dominant-negative TBX2 construct resulted in up-regulation of p19^{ARF}, providing strong support for a model, in which loss of SAFB1 causes an increase in TBX2 levels that in turn down-regulate p19^{ARF} promoter activity. The question remains as how SAFB1 regulates TBX2 expression. Preliminary experiments in our laboratory failed to establish a direct link in transient assays, suggesting a more complicated mechanism, which will be the focus of our future studies. An interesting possibility is that SAFB1 regulates TBX2 expression through effects on chromatin organization because the TBX2 5'-regulatory region contains a S/MAR, a consensus element that can be recognized by SAFB1.

Immortalization requires either biallelic loss of p19^{ARF} or inactivation of p53. For instance, p19^{ARF} heterozygous MEFs do not immortalize unless they lose the second allele (19). Interestingly, SAFB1^{-/-} MEFs are able to immortalize despite incomplete loss of p19^{ARF} expression. This suggests that loss of SAFB1 causes additional changes in as-yet-unknown target genes that cooperate with low p19^{ARF} levels in immortalization. Such genes are unlikely to be downstream effectors of p19^{ARF} (including p53) because reintroduction of p19^{ARF} into SAFB1^{-/-} MEFs readily inhibits cell proliferation, as one would expect from a functional p19^{ARF}-p53 pathway.

Previous work from the DePinho group has established that p19^{ARF} and p16INK4a loss do not cooperate with T antigen in MEF transformation (21). Therefore, our findings that loss of SAFB1 cooperates with T antigen support the notion from above that loss of SAFB1 may cause additional p19^{ARF}-independent alterations.

Collectively, our data suggest that loss of SAFB1 facilitates immortalization of primary MEFs and increased cell transformation. Our study is particularly important because it connects a gene involved in breast tumorigenesis to the senescence process. Ongoing and future experiments will determine whether the mechanism(s) by which SAFB1 interferes with the senescence program in MEFs is also conserved in human cells.

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References

- Oesterreich S. Scaffold attachment factors SAFB1 and SAFB2: innocent bystanders or critical players in breast tumorigenesis? *J Cell Biochem* 2003;90:653-61.
- Townson SM, Dobrzycka KM, Lee AV, et al. SAFB2, a new scaffold attachment factor homolog and estrogen receptor corepressor. *J Biol Chem* 2003;278:20059-68.
- Nayler O, Stratling W, Bourquin JP, et al. SAF-B protein couples transcription and pre-mRNA splicing to SAR/MAR elements. *Nucleic Acids Res* 1998;26:3542-9.
- Weighardt F, Cobianchi F, Cartegni L, et al. A novel hnRNP protein (HAP/SAF-B) enters a subset of hnRNP complexes and relocates in nuclear granules in response to heat shock. *J Cell Sci* 1999;112:1465-76.
- Kipp M, Gohring F, Ostendorp T, et al. SAF-Box, a conserved protein domain that specifically recognizes scaffold attachment region DNA. *Mol Cell Biol* 2000;20:7480-9.
- Townson SM, Kang K, Lee AV, Oesterreich S. Structure-function analysis of the estrogen receptor α corepressor scaffold attachment factor-B1: identification of a potent transcriptional repression domain. *J Biol Chem* 2004;279:26074-81.
- Oesterreich S, Zhang Q, Hopp T, et al. Tamoxifen-bound estrogen receptor (ER) strongly interacts with the nuclear matrix protein HET/SAF-B, a novel inhibitor of ER-mediated transactivation. *Mol Endocrinol* 2000;14:369-81.
- Oesterreich S, Allred DC, Mohsin SK, et al. High rates of loss of heterozygosity on chromosome 19p13 in human breast cancer. *Br J Cancer* 2001;84:493-8.
- Ivanova M, Dobrzycka KM, Jiang S, et al. Scaffold attachment factor b1 functions in development, growth, and reproduction. *Mol Cell Biol* 2005;25:2995-3006.
- Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995;83:993-1000.
- Bollag RJ, Siegfried Z, Cebra-Thomas JA, et al. An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T locus. *Nat Genet* 1994;7:383-9.
- Vance KW, Carreira S, Brosch G, Goding CR. Tbx2 is overexpressed and plays an important role in maintaining proliferation and suppression of senescence in melanomas. *Cancer Res* 2005;65:2260-8.
- Sherr CJ. The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol* 2001;2:731-7.
- Jacobs JJ, Kieboom K, Marino S, DePinho RA, van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature* 1999;397:164-8.
- Gil J, Bernard D, Martinez D, Beach D. Polycomb CBX7 has a unifying role in cellular lifespan. *Nat Cell Biol* 2004;6:67-72.
- Jacobs JJ, Keblusek P, Robanus-Maandag E, et al. Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. *Nat Genet* 2000;26:291-9.
- Lingbeek ME, Jacobs JJ, van Lohuizen M. The T-box repressors TBX2 and TBX3 specifically regulate the tumor suppressor gene p14ARF via a variant T-site in the initiator. *J Biol Chem* 2002;277:26120-7.
- Hanahan D, Weinberg RA. The hallmarks of cancer. *Cell* 2000;100:57-70.
- Kamijo T, Zindy F, Roussel MF, et al. Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell* 1997;91:649-59.
- Lowe SW, Sherr CJ. Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev* 2003;13:77-83.
- Pomerantz J, Schreiber-Agus N, Liegeois NJ, et al. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* 1998;92:713-23.